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14. ABSTRACT

The goal of my DOD-supported research is determine the role of the new mTOR complex (mTORC2) in Autism Spectrum Disorder (ASD). ASD individuals exhibit impaired social interactions, seizures and abnormal repetitive behavior. In addition, 70-80% of autistic individuals suffer from mental retardation. Autism is a heritable genetically heterogeneous disorder and mutations in negative regulators of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, such as PTEN were associated with ASD. Here, we show that in the hippocampus of Pten fb-KO mice – where Pten is conditionally deleted in the murine forebrain – the activity of both mTORC1 and mTORC2 is increased. In addition, Pten fb-KO mice exhibit seizures, learning and memory, stereotype/repetitive behaviors and social deficits. Our remarkable preliminary data show that genetic inhibition of mTORC2, but not mTORC1, in Pten-deficient mice significantly promotes survival and seizures. In addition, Pten-rictor fb- double KO (DKO) mice, in which mTORC2 activity is restored to normal levels, EEG seizures, learning and memory as well as social phenotypes, are all rescued. We also found that in Pten-deficient mice mitochondria respiration is impaired and mTORC2 silencing restores mitochondria dynamics. In the third, year we will investigate the mechanism by which mTORC2 regulates mitochondria respiration. In addition, we will assess metabolic changes in neurons lacking Pten. These insights hold the promise for new mTORC2-based treatment of ASD.

15. SUBJECT TERMS
Autism Spectrum Disorder (ASD), mTORC2, mTORC1, protein synthesis, actin polymerization, mitochondria function, long-term memory, social behaviors, repetitive behaviors, and seizures.

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1. Introduction:

Autism represents a heterogeneous group of disorders, which are defined as "autism spectrum disorders" (ASDs). ASD individuals exhibit common features such as impaired social interactions, language and communication, and abnormal repetitive behavior. In addition, 70-80% of autistic individuals suffer from mental retardation[1-3]. The major goal of this award is to determine the role of mTORC2 in two mouse models of ASD. Recently, we have shown that mTORC2 plays a crucial role in long-term memory formation[4]. Briefly, mice lacking mTORC2 showed impaired long-lasting changes in synaptic strength (L-LTP) as well as impaired long-term memory (LTM). In addition, we have found that by promoting mTORC2 activity, with a new agent A-443654, it facilitates L-LTP and enhances long-term memory formation in WT mice. Interestingly, mTORC2 activity is altered in both ASD patients and ASD mouse models harboring mutation in *Tsc* and *Pten*[5,6]. Hence, in this proposal we will test the hypothesis that the neurological dysfunction in several ASD mouse models is caused by dysregulation of mTORC2 rather than mTORC1 activity.

2. Keywords: Autism Spectrum Disorder (ASD), mTORC2, mTORC1, protein synthesis, actin polymerization, mitochondria function, long-term memory, social behaviors, repetitive behaviors, seizures.

3. Overall Project Summary. We have generated a remarkable amount of progress regarding specific Aim 2. The current view posit that in Pten-deficient mice increased mTORC1 activity is responsible for the behavioral, electrophysiological and pathophysiological abnormalities in these mice[7]. However, mTORC2 activity is also increased in Pten-deficient mice[8,9]. Thus, we wondered whether reduction of either mTORC1 or mTORC2 could rescue the ASD-like cellular and behavioral endophenotypes in PTEN-deficient mice. To this end, we used genetic and conditionally deleted a) Pten and/or rictor, a defining mTORC2 component[6], or b) Pten and/or raptor, a defining mTORC1 component, in the murine forebrain using the Cre/lox system. Because the αCaMKII promoter is inactive before birth[10,11], this manipulation rules out possible developmental defects caused by the lack of pten, *rictor or raptor*. We thus generated and studied the followings experimental mice: WT mice, *Pten* forebrain-specific knockout (here defined as *Pten* fb-KO), *Pten-rictor* forebrain-specific double knockout (here defined as *Pten-Rictor* fb-DKO) and Pten-raptor forebrain-specific KO (Pten-Raptor fb-DKO).

Specific inhibition of mTORC2 and mTORC1 activities in Pten-deficient mice. According to our preliminary data, mTORC2-mediated phosphorylation of Akt at Ser473 (an established readout of mTORC2 activity[6]) and mTORC1-mediated phosophourylation of ribosome protein S6 (an established readout of mTORC1 activity)[12] were both increase in the hippocampus of *Pten* fb-KO. By contrast, in *Pten-Rictor* fb-DKO, while mTORC1 activity is up-regulated, mTORC2 activity is suppressed. In addition, in *Pten-Raptor* fb-DKO, mTORC2 activity is up-regulated by mTORC1 activity is suppressed. Hence, conditional deletion of rictor selectively blocks mTORC2 activity in Pten-deficient neurons and conditional deletion of raptor selectively block mTORC1 activity in Pten-deficient neurons.

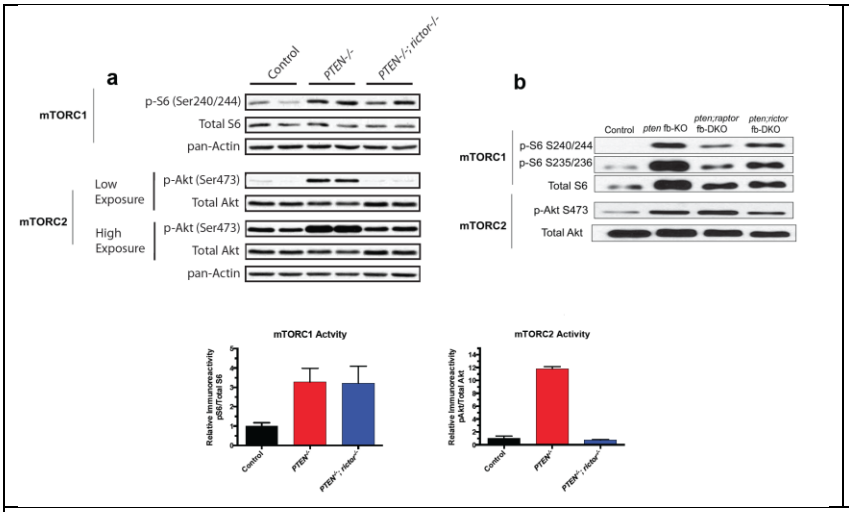

Fig. 1. mTORC2 activity is selectively suppressed in the hippocampus from *Pten-rictor* fb-KO mice, whereas mTORC1 activity is selectively suppressed in the hippocampus of Pten-raptor fb-DKO. a, b. Western blots reveals that mTORC1 activity (p-S6 Ser240/244) is unaltered in the brain of *Pten-rictor* fb-DKO mice compared to *Pten* fb-KO mice (n=5) but mTORC2 activity is suppressed. By contrast, mTORC2 activity is unaltered in the brain of Pten-raptor fb-DKO mice compared to *Pten* fb-KO mice, but mTORC1 activity is reduced. Bottom: A significant decrease in mTORC2 activity (p-Akt-Ser-473) is observed in the hippocampus of *Pten-rictor* fb-KO mice, compared to *Pten-rictor* fb-KO mice.

<u>Genetic inhibition of mTORC2, but not mTORC1 prolonged survival in Pten-deficient mice.</u> Kaplan-Meier analysis of animal survival revealed a dramatic decreased in survival in *Pten* fb-KO mice (**Fig. 2).** Genetic elimination of raptor had no effect on *Pten* fb-KO mice survival (compare *Pten* fb-KO mice vs. *Pten-Raptor* DKO survival curves). Remarkably, genetic elimination of rictor significantly prolonged survival in Pten-defiicient mice (compare *Pten* fb-KO mice vs. *Pten-Rictor* DKO survival curves**; Fig. 2;** *Pten-Rictor* fb-DKO die at an age of 119.4 +/- 25). Hence, inhibition of mTORC2, but not mTORC1, in Pten-deficient mice prolongs their survival.

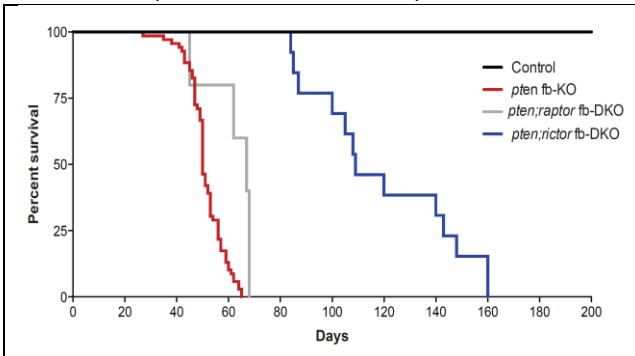

Fig. 2. Genetic inhibition of mTORC2 prolongs survival in *Pten*-deficient mice. Kaplan-Meier survival curves for wild-type, Pten fb-KO, Pten-raptor fb-DKO and Pten-rictor fb-DKO. The median survival for *Pten-rictor* fb-DKO (n=35) is 120 days, a 140% extension in life span compared with the single Pten- fb-DKO mice (n=11) or Pten-raptor fb-DKO (n=8). The curves of *Pten* fb-KO mice and *Pten-rictor* DKO mice are significantly different (p<0.0001).

<u>Genetic inhibition of mTORC2, but not mTORC1 supressed seizures in Pten-deficient mice.</u>

Because patients with Pten mutations exhibit seizures, we next analyzed spontaneous seizures and abnormal electroencephalogram (EEG) activity. We found that *Pten* fb-KO mice show abnormal EEG as well as tonic-clonic seizures (**Fig. 3**). Consistent with the survival curves, Pten-raptor fb-DKO mice also showed tonic-clonic and EEG seizures. By contrast, while *Pten-Rictor* fb-DKO showed some abnormalities (spikes) in the EEG pattern, they did not show tonic-clonic and/or EEG seizures (**Fig. 3**). Thus, inhibition of mTORC2, but not mTORC1, suppresses the seizures EEG phenotype in Pten-deficient mice. Because in Pten-raptor fb-DKO mice genetic

inhibition of mTORC1 had not effect on seizures onset and duration and animal survival, in future experiments we focused on the characterization of Pten-rictor deficient mice.

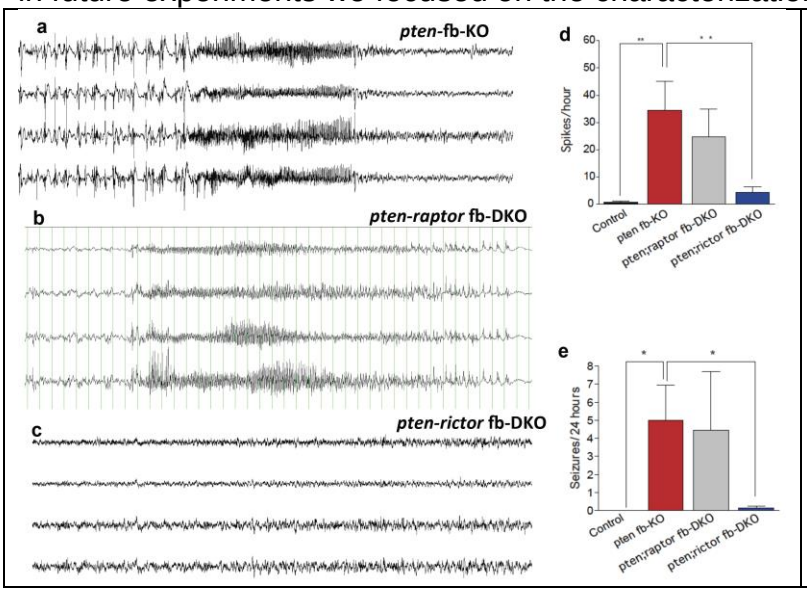

Fig. 3. Genetic inhibition of mTORC2, but not mTORC1, suppresses EEG seizures *Pten* fb-KO mice. Traces from bilateral cortical electrodes (Left hemisphere, L, Right hemisphere, R) show abnormal EEG seizures in freely moving *Pten* fb-KO mice (**a;** n=6) and Pten-raptor fb-DKO mice (**b,** n=3). In *Pten-rictor* fb-DKO mice EEG seizures are suppressed which are suppressed in (**c;** n=5). d,e, Quantification of spikes and seizure frequencies.

Genetic inhibition of mTORC2 rescues LTM deficits in Pten-deficient mice.

Since emotions have a powerful impact on memory – most vivid autographical memories tend to be of emotional effects, and 70-80% of autistic individuals suffer from mental retardation [1-3], we first examined emotional memory. To this end, mice were first studied in contextual Pavlovian fear conditioning. Contextual fear conditioning, and hippocampus-dependent task, was induced by pairing a context (conditioned stimulus; CS) with a foot shock (the unconditioned stimulus; US). Mice were subsequently exposed to the auditory tone or visual stimulus (CS) and fear responses [mouse stop moving ("freezes")] were taken as an index of the strength of memory (**Fig. 4a**). As expected, compared to control mice, *Pten* fb-KO mice showed impaired long-term fear memory (**Fig. 4b).** Strikingly, long-term memory is significantly improved in *Pten-rictor* fb-DKO mice. Thus, silencing mTORC2 activity restores LTM in Pten-deficient mice.

We next studied object recognition, another hippocampal dependent task. In this task, an object is presented to the subject mouse. After a 24 hr delay, the object is presented again with a new object (**Fig. 4c).** The time spent exploring each object is tracked via by a computer-operated optical animal activity system (ANIMAZE). We found that Pten-deficient mice failed to discriminate between and old and a new object (**Fig. 4d**). However, genetic deletion of rictor restore the object recognition long-term memory deficits in Pten-deficient mice.

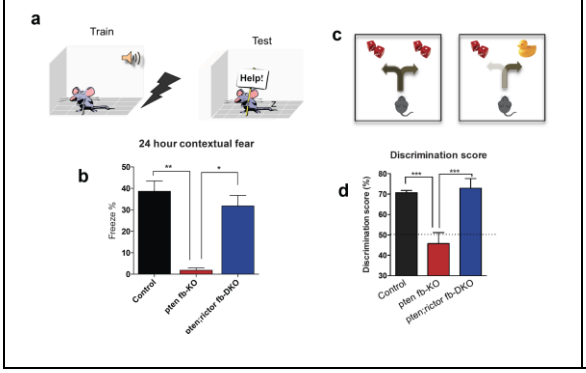

Fig. 4. Genetic inhibition of mTORC2 rescues the deficient long-term fear and object recognition memory in Pten fb-KO mice. a, Schematic of experimental design. **b,** For contextual fear conditioning, freezing times were recorded 24 hr after conditioning. As compared to control mice, Pten fb-KO mice show deficient freezing 24 hr after training, indicating that their long-term memory is impaired. In Pten-rictor fb-DKO mice, freezing levels are similar to those observed in WT mice, indicating that their long-term memory is restored. **c,** Schematic of object recognition task. **d)** Discrimination scores between two objects.

Genetic inhibition of mTORC2 rescues social behaviors in Pten-deficient mice.

Given that social interaction deficits are salient features of ASD individuals[2], we next studied social behaviors. First, we assessed reciprocal social interactions by recording the amount of time a pair of mice spent interacting in a neutral arena[13] (**Fig. 5a**). We found that compared to control mice, Pten-deficient mice showed reduced reciprocal interaction (**Fig. 5b**). Interestingly, in Pten-rictor DKO mice, reciprocal social interaction is normal. Next, we measured sociability and preference for social novelty using the Crawley 3-chamber test[13] (**Fig. 5c, 5e**). In the sociability task, we compared the time a mouse spends interacting with an empty wired cage and one containing a mouse (**Fig. 5c**); whereas in the social novelty test, we measured the time a mouse spends interacting with a familiar or a stranger mouse (**Fig. 5e**). Consistent with the direct social interaction results, we found that Pten fb-KO mice had normal sociability (**Fig. 5d**), but showed no preference for interaction with a stranger versus a familiar mouse in the social novelty test (**Fig. 5f**). Strikingly, deletion of mTORC2 rescues the social novelty deficits in Pten-deficient mice (Fig. 5f). Taken together these data indicate that Pten-fb KO mice display social deficits and inhibition of mTORC2 restored their phenotypes.

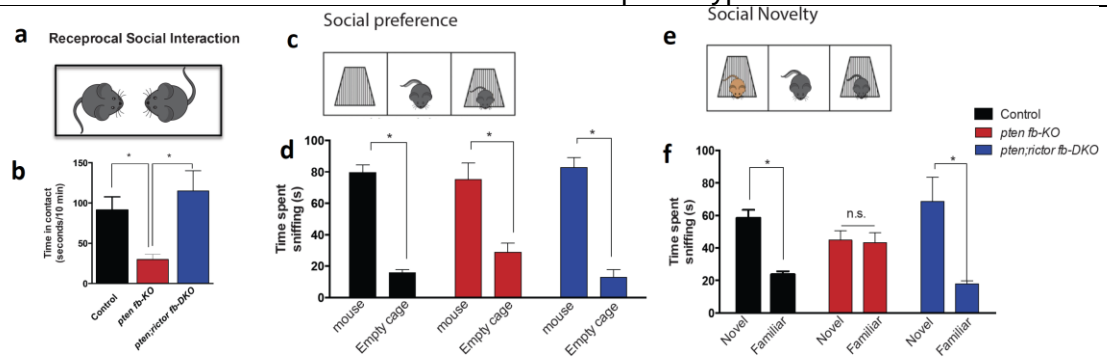

Fig. 5. Social behaviors are normal in Pten-rictor fb-DKO mice. a) Schemetic of reciprocal social interaction task**. b,** Compared to control or pten-rictor fb-DKO mice, pten-fb KO mice showed reduced reciprocal interaction. **c, e,** Schematic of three chamber social interaction task. **f,** Control mice spent most of the time interacting with the stranger mouse but *Pten* fb-KO mice spent equal time interacting with the familiar or strange mouse, indicating that social behavior is impaired in these mice. Like WT mice, *Pten-rictor* fb-KO mice spent more time interacting with the stranger mouse, indicating that social behavior is restored.

Genetic inhibition of mTORC2 rescues repetitive behaviors in Pten-deficient mice.

Because ASD patients also exhibit repetitive/stereotyped behavior behaviors, we first assessed marble burying. In this task, mice were individually placed in Plexiglas cages containing 5 cm deep fresh bedding, with 20 black marbles pre-arranged in 5x4 evenly spaced rows (**Fig. 6a**). Testing was conducted for 20 min. After the test period, unburied marbles were counted. We found that Pten-deficient mice buried more marbles than control mice, indicating repetitive behavior (Fig. 6b). Remarkably, deletion of rictor restored the repetitive behavior in Pten-deficient mice (Fig. 6b). We next assessed behavioral flexibility in the T-maze. In this task, normal animals tend to investigate first one and the other arm of the maze. Notably, Pten-deficient mice explored the same arm of the maze, demonstrating and impairment in behavioral flexibility. Remarkably, in Pten-rictor fb-DKO mice restored this behavior. Thus, taken together inhibition of mTORC2 restore memory, social and repetitive behaviors in Pten-deficient mice.

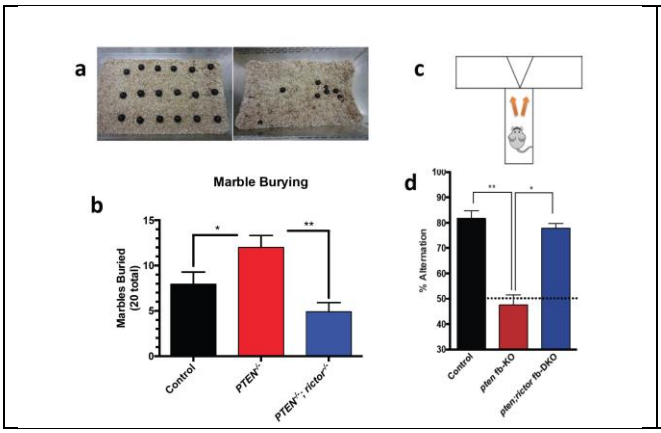

Fig. 6. Behavioral flexibility and repetitive behaviors are restored by deletion of mTORC2 in Pten-deficient mice.

Genetic inhibition of mTORC2 restores mitochondria respiration in Pten-deficient mice.

Given that a) mitochondria dysfunction has been found in ASD patients[14-16], b) Pten mutation affect mitochondria function[17] and c) mTORC2 has been found in mitochondria[18], we studied mitochondria respiration. We found that mitochondria respiration is impaired in Pten-deficient mice and genetic inhibition of mTORC2 restored it (**Fig. 7**). We will now investigate the precise mechanism by which mTORC2 silencing restores mitochondria function.

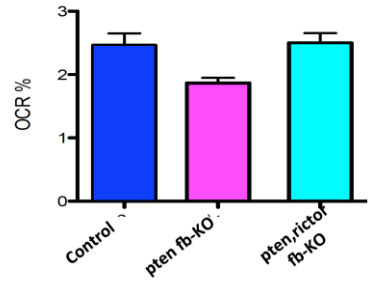

Fig. 7 Mitochondria respiration is restored in Pten-rictor deficient mice. Oxygen consumption rate (OCR) is reduced in Pten-deficient mice, but restored when mTORC2 activity is genetically inhibited.

The goal of subaim-2 was to increase mTORC2 activity by treating Tsc2-deficient mice with the mTORC2 agonist A-443654[4]. We have obtained Tsc2 floxed mice but after breeding we noticed that they were in a mix background. Hence, to avoid any behavioral artifacts due to mixed genetic background, we decided to backcross these mice to C57BL6 mice to obtain a pure backgound. It is noteworthy that genetic background may alter behavior of autistic mice[19]. At least 8-10 generations are required to obtain mice in a pure background. We have so far backrossed our mice 6 generation and in two generation we will cross our mice with CamKII-Cre. We will next treat the conditionalTsc2-deficient mice with vehicle and A-443654 and behavior and electrophysiology will be performed in these mice, as discussed in the proposal.

4. Key Research Accomplishment

- We developed a way to specifically block mTORC2 activity in Pten-deficient mice.
-We developed a way to specifically block mTORC1 activity in Pten-deficient mice
- Genetic deletion of mTORC2 prolongs the survival of Pten-deficient mice.
- Genetic deletion of mTORC2 dramatically attenuates seizures in Pten-deficient mice.
- Genetic deletion of mTORC2 improves cognitive and social phenotypes in Pten-deficient mice.
- Genetic deletion of mTORC2 improves repetitive behaviors.
- Genetic deletion of mTORC1 failed to restore animal's survival and seizures phenotype.

5. Conclusion

It has been proposed that the increased mTORC1 in Pten-deficient or Tsc-deficient mice causes the cellular and behavioral phenotypes associated with ASD[20-26]. Our new data challenge this view and provide causal evidence that inhibition of mTORC1 had no effect on Pten-induced physiopathology. More importantly, our data demonstrate that the neurological dysfunction in ASD, at least in the Pten-ASD mouse model, is caused by dysregulation of mTORC2. Hence, these preliminary data are very important since they identified a new signaling pathway involved in ASD and seizure disorders that could be targeted and lead to the development of new treatments for ASD and seizure disorders.

Future experiments: We will study the precise mechanism by which mTORC2 suppresses Pten-mediated cellular and behavioral phenotypes. We will focus on the mechanism by mTORC2 restores mitochondria respiration in Pten-deficient mice. Our preliminary experiments show that in Pten-deficient mice mitochondria respiration is reduced and deletion of mTORC2 restore its function. We will perform metabolomics in the hippocampus from Pten-deficient and Pten-rictor fb-DKO mice. Finally, we will start to characterize the vehicle-treated and A-443654-treated Tsc-deficient mice in behavior and electrophysiology.

6. Publications

Some of these data described above were presented in
- "Catastrophic Epilepsy" at the Neurological Research Institute (NRI), Houston, Texas
- Symposium Society for Neuroscience (see 1: Huber KM, Klann E, Costa-Mattioli M, Zukin RS. Dysregulation of Mammalian Target of Rapamycin Signaling in Mouse Models of Autism. J Neurosci. 2015 Oct 14;35(41):13836-42. doi: 10.1523/JNEUROSCI.2656-15.2015. PubMed PMID: 26468183; PubMed Central PMCID: PMC4604222.)

7. Inventions, Patents and Licenses.

Nothing to report

8. Reportable Outcome

Nothing to report

9. Other achievements

We developed forebrain-specific rictor-Pten double KO mice.
We developed forebrain-specific raptor-Pten double KO mice.

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11. Appendices

N/A